Reference: *Biol. Bull.*, 146: 404–414. (June, 1974)

STUDIES ON THE BIOLOGICAL PROPERTIES OF COELOMIC FLUID OF SEA URCHIN. II. NATURALLY OCCURRING HEMAGGLUTININ IN SEA URCHIN

KAZUO RYOYAMA

Department of Experimental Therapeutics, Cancer Research Institute, Kanazawa University, Kanazawa (Japan)

It is well known that invertebrates may have hemagglutinins for a variety of erythrocytes (Huff, 1940; Tyler, 1946; Cushing, Calaprice and Trump, 1963; Brown, Almodovar, Bhatia and Boyd, 1968; Prokop, Uhlenbruck and Köhler, 1968; McKay, Jenkin and Rowley, 1969).

Recently, in the course of studies on the biological properties of coelomic fluid from sea urchins (*Anthocidaris crassipina*, *Pseudocentrotus depressus*, and *Hemicentrotus pulcherrimus*), it was demonstrated that the fluid was highly effective to lyse erythrocytes from some species of animals and that the nature of its active principle may be a protein or a protein-like substance (Ryoyama, 1973). Soon afterward, it was found that the same materials from these sea urchins also exhibited hemagglutinating activity against rabbit erythrocytes. Although natural hemagglutinin can be found in the coelomic fluid of sea urchin, its chemical and biological properties have been poorly understood (Tyler, 1946; Brown, *et al.*, 1968; Uhlenbruck, Sprenger and Heggen, 1970). The present report gives data on the occurrence of hemagglutinins in the coelomic fluid of sea urchins stated above, and on some properties of their hemagglutinins.

Materials and Methods

Three species of sea urchin, *Anthocidaris crassispina* (Agassiz), *Pseudocentrotus depressus* (Agassiz), and *Hemicentrotus pulcherrimus* (Agassiz) were used. They were harvested at three different littoral areas (Ogi and Kabuto in Ishikawa Prefecture, and Amabarashi in Toyama Prefecture, Japan). The coelomic fluid preparation was obtained by the method reported previously (Ryoyama, 1973). The fluid (protein content, 467 μg/ml) pretreated at 56° C for 30 minutes to suppress their hemolytic activity was used throughout the experiments, except for heat stability test of the hemagglutinating activity. The protein content of the coelomic fluid preparation was determined by the Folin-phenol method (Lowry, Rosebrough, Farr, and Randall, 1951), using bovine albumin as a reference standard.

The erythrocytes from various animals were centrifuged at $250 \times g$ and suspended in 0.05 M Tris-HCl buffer solution (pH 8.0) containing 0.15 M NaCl and 0.02 M $CaCl_2$ (Tris-NaCl-$CaCl_2$ buffer solution). Unless otherwise stated, this buffer solution was used for dialyzing coelomic fluid, diluting the dialyzed fluid preparation, and also for suspending erythrocytes. The final concentration of erythrocytes was adjusted to $1.0–1.6 \times 10^8$ cells per ml.

To serial two-fold dilutions of the coelomic fluid preparation, an equal volume

TABLE I

Relative hemagglutinating activity of coelomic fluid preparation obtained from three different species of sea urchin against erythrocytes from various species of animals

Erythrocyte species	Hemagglutinating titer*		
	A. crassispina	*P. depressus*	*H. pulcherrimus*
Human-Blood group A	2^5	2^3	2^3
B	2^4	2^4	2^4
O	2^3	2^2	2^3
Rabbit	2^9	2^6	2^9
Mouse	<2	<2	<2
Guinea pig	<2	2^3	2^5
Dog	2^6	<2	2^6
Sheep	<2	<2	<2

* Represented as the reciprocal of the highest dilution effecting agglutination.

of erythrocyte suspension was added. After mixing, each of the test tubes was incubated at 37° C for 30 minutes and hemagglutination was examined macroscopically or microscopically.

Trypsin (Difco, 1 : 250) and Bromelains (from pineapple) from the Nutritional Biochemical Corp. were used.

RESULTS

Effect of coelomic fluid preparation against erthyroctes from different species of animals

The results obtained in the experiments to examine the susceptibility of erythrocytes from different sources to coelomic fluid preparation from sea urchin are presented in Table I. Rabbit erythrocytes were found to agglutinate when they were mixed with the coelomic fluid preparation. Hemagglutinin titers of *A. crassispina* and *H. pulcherrimus* against rabbit cells were 2^8 to 2^9 and that of *P. depressus* was 2^4 to 2^6. The coelomic fluid from *H. pulcherrimus* reacted more actively than those from the other two species against rabbit cells, though the titers of agglutination by *H. pulcherrimus* and *A. crassispina* were almost the same. Human erythrocytes were also sensitive to these coelomic fluid preparations, and their hemagglutinating activities were much the same. However, some differences in susceptibility against the fluids were found among the type of blood group (A, B, O) in man; A- and B-type cells were more sensitive than O-type cells. The buffer solution itself used in these experiments had no effect on erythrocytes. There were some variations in agglutinating activity among species of sea urchin. The coelomic fluid preparation from *A. crassispina* agglutinated dog erythrocytes but that from *P. depressus* did not. Conversely, the fluid from *P. depressus* agglutinated guinea pig erythrocytes but that from *A. crassispina* did not. The fluid from *H. pulcherrimus* equally produced hemagglutination of erythrocytes from both animals. On the other hand, three coelomic fluid preparations did not hemagglutinate either sheep or mouse erythrocytes.

TABLE II

Effect of bivalent cations on the hemagglutinating activity of 3 coelomic fluid preparations against rabbit erythrocytes

Final concentration of metallic salt	Hemagglutinating titer		
	A. crassispina	*P. depressus*	*H. pulcherrimus*
None	$<2^2$	$<2^2$	$<2^2$
10 mM $CaCl_2$	2^7	2^6	2^7
20 mM $CaCl_2$	2^8	2^6	2^8
100 mM $CaCl_2$	2^8	2^6	2^8
20 mM $MgCl_2$	$<2^2$	$<2^2$	$<2^2$
20 mM $MgCl_2$ + 20 mM $CaCl_2$	2^7	2^6	2^7
20 mM $CaCl_2$ + 20 mM EDTA	$<2^2$	$<2^2$	$<2^2$
40 mM $CaCl_2$ + 20 mM EDTA	2^7	2^6	2^6

In addition, hemagglutinating activity of coelomic fluid was not dependent on age (size of shell) or sex of sea urchin, but might be dependent on the season of collection; in the case of *A. crassispina*, all the specimens collected during April and May showed hemagglutinating activity, a few of the specimens collected during July and August did not show this activity, and only a few of the specimens collected in November showed this activity. (The spawning season of this species is June to August). Moreover, hemagglutinating spectra of coelomic fluids from specimens collected in different places were also similar to that presented in Table I.

The sea water of the place where sea urchin was collected was almost entirely ineffective to erythrocytes, even when concentrated sea water was used.

Furthermore, no difference in the hemagglutinating potency of the coelomic fluid preparation was observed in the range of 1° C to 37° C and pH 6.6 to 9.3. Therefore, it may be said that exhibition of hemagglutinination by the fluid preparation is not dependent on temperature. On the other hand, the agglutinating activity of the fluid from *P. depressus* was found to be slightly higher at 4° C than at 37° C against guinea pig erythrocytes.

Since rabbit cells among the tested erythrocytes were found to be the most susceptible to coelomic fluid preparation from sea urchin, the following experiments were carried out mainly on rabbit cells.

Effect of calcium on the hemagglutinating activity of coelomic fluid preparation

A preliminary experiment had demonstrated that calcium ion brought a marked increase of hemagglutinating titer of coelomic fluid preparation. Further investigations on the enhancing effect of calcium ion was repeated employing the following system: A mixture of 0.1 ml of coelomic fluid preparation (dialyzed against 0.85% NaCl solution), which was diluted two-fold serially with 0.85% NaCl solution, 0.1 ml of rabbit erythrocytes suspended in Tris-NaCl buffer solution, and 0.2 ml metallic salt in the same buffer solution was incubated at 37° C for 30 minutes.

As shown in Table II, addition of $CaCl_2$ to this hemagglutination test system greatly increased the activity of coelomic fluid preparation. No agglutination was

observed in the absence of coelomic fluid preparation or in the presence of $CaCl_2$ alone. The addition of EDTA abolished this effect, which was regained on further addition of calcium. Magnesium had no effect on hemagglutination and also did not interfere with the enhacing effect of calcium. Enhancement of hemagglutinating activity of coelomic fluid preparation by calcium ion was also observed in the experiment with guinea pig and human erythrocytes.

It thus appears that calcium is essential for hemagglutination produced by coelomic fluid preparation from three different species of sea urchin.

Effect of temperature and pH on the stability of coelomic fluid preparation

In order to examine the effect of temperature on the stability of hemagglutinating activity of coelomic fluid preparation, aliquots of the fluid were placed in a water bath in the range of 1 to 100° C for 30 minutes, and hemagglutinating activity was then estimated. In the case of *A. crassispina*, hemagglutinating activity of the coelomc fluid preparation was not affected in the temperature range of 1 to 65° C, while its activity was almost completely destroyed at 70° C. In the case of *P. depressus*, the activity was not affected to 70° C, but was destroyed (about 50%) at 75 or 80° C, and completely abolished at 85° C. On the other hand, the coelomic fluid preparation from *H. pulcherrimus* was not affected even at 100° C.

When the coelomic fluid preparations dialyzed against 0.85% NaCl solution were placed at 4° C for 12 hours in the buffer solution of various pH values (3.2 to 10.6), no change of the activity was observed at the the pH range stated above. The coelomic fluid preparations from three different species of sea urchin seem to be very stable in the region of these pH values.

Effect of some agents on the stability of coelomic fluid preparation

The coelomic fluid preparations from the three species were digested by proteinase (trypsin, bromelain) in order to ascertain whether or not the factor carring the hemagglutinating activity of each fluid was a protein. The reaction mixture of 0.3 ml of coelomic fluid preparation and 0.3 ml of trypsin solution (1–5 mg/ml) or bromelain solution (0.1–1 mg/ml) was incubated at 37° for 1 hour. To each of the reaction mixtures diluted serially was added an equal volume of erythrocyte suspension, and the mixture was placed at 4° C for 30 minutes to minimize the enzyme action. As the results, trypsin had no effect on hemagglutinating activity. No hemagglutination was observed in the absence of coelomic fluid preparation or in the presence of trypsin alone. Accordingly, hemagglutinating activity of the fluids from three species of sea urchin seems to be resistant to tryptic digestion, while bromelain at 0.1 or 0.05% concentrations brought about a considerable decrease in the activity when the coelomic fluid preparations from *A. crassispina* and *P. depressus* were used. On the other hand, the fluid from *H. pulcherrimus* was not affected by bromelain under the same condition. No hemagglutinination was observed in the presence of bromelain alone. In addition, susceptibility of the rabbit erythrocytes pretreated with bromelain or typsin to coelomic fluid preparation was not impaired but slightly increased. Thus, it was thought that the decrease in the activity was actually due to digestion of the coelomic fluid (from *A. crassispina* and *P. depressus*) by bromelain.

TABLE III

Effect of 2-mercaptoethanol on the hemagglutinating activity of 3 coelomic fluid preparations against rabbit erythrocytes

Concentration (M)	Hemagglutinating titer		
	A. crassispina	*P. depressus*	*H. pulcherrimus*
—	3×2^7	3×2^3	3×2^5
0.2	3×2^7	3×2^2	3×2^4
0.4	$<3 \times 2$	$<3 \times 2$	3×2^2
0.4*	3×2^7	$<3 \times 2$	3×2^4

* Without treatment with sodium iodoacetate.

Further experiment was made to investigate whether or not 2-mercaptoethanol, which is a reagent that breaks up the S-S linkage in protein molecules, affects hemagglutinating activity of coelomic fluid preparation. The experiment was carried out as follows: A mixture of 0.3 ml of 2-mercaptoethanol (0.4–0.2 M) and 0.3 ml of coelomic fluid preparation was incubated at 17° C for 1 hour. To each of the reaction mixtures was added 0.3 ml of sodium iodoacetate solution (0.6–0.3 M), and then placed at 4° C for 12 hours, followed by dialysis against Tris-NaCl-$CaCl_2$ buffer solution. These results are shown in Table III. Hemagglutinating activity of coelomic fluid preparations (from *A. crassispina* and *P. depressus*) was completely destroyed by 0.4 M of 2-mercaptoethanol, but the fluid from *H. pulcherrimus* was slightly affected at the same concentration of the reagent. When the fluids pretreated with 2-mercaptoethanol were dialyzed against Tris-NaCl-$CaCl_2$ buffer solution without iodoacetate treatment, hemagglutinating activity of the fluid from *A. crassispina* was completely restored, but not that of the fluid from *P. depressus*. The treatment with sodium iodoacetate alone did not affect the activity. The effect of periodate on the fluid preparation was examined as follows: A mixture of 0.3 ml of coelomic fluid preparation dialyzed against 0.85% NaCl solution, 0.2 ml of 0.2 M citrate buffer (pH 5.5), and 0.1 ml of various concentration of periodate solution adjusted to pH 5.5 with 2 N NaOH was incubated at 20° C for 4 hours. The reaction mixture was dialyzed overnight against 0.85% NaCl solution and then against Tris-NaCl-$CaCl_2$ buffer solution to remove the periodate, and the activity was estimated. Periodate is known to destroy sugars by oxidation. Hemagglutinating activity of the fluid preparation from *H. pulcherrimus* was not affected even with 100 mM of periodate, whereas that of *A. crassispina* and *P. depressus* was completely destroyed at 25 mM of the reagent. The activity of coelomic fluid treated at these conditions without periodate was not changed.

Inhibition tests with simple sugars

In an attempt to find the chemical nature of specific receptor of sea urchin agglutinins, inhibition tests were carried out with 36 simple sugars. These particular sugars have been generally used to examine the specificity of agglutinins. A number of authors have reported that the specificity of most receptor sites against

various agglutinins depends to some degree on the sugar moiety in receptor molecules. From each of these sugars, 0.2 M stock solution in Tris-NaCl-$CaCl_2$ buffer solution was prepared, with the exception of dulcitol, salicin, and raffinose in 0.067 M, and cellobiose, lactose, and D-melezitose in 0.1 M. Equal volumes of the coelomic fluid preparation were added to each serial dilution of the stock sugar solution, and incubated at 20° C for 2 hours. After addition of rabbit erythrocyte suspension, the incubation mixture was reincubated at 37° C for 30 minutes. Test materials of mono-, oligo-, and derived saccharides included D-ribose, 2-deoxy-D-ribose, D-arabinose, L-arabinose, D-xylose, L-xylose, D-glucose, L-glucose, 2-deoxy-D-glucose, D-mannose, D-galactose, D-sorbitol, D-mannitol, dulcitol, inositol, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, N-acetyl-D-galactosamine, D-glucosamine HCl, D-mannosamine HCl, D-galactosamine HCl, salicin, L-rhamnose, D-digitoxose, D-fucose, L-fucose, D-fructose, L-sorbose, cellobiose, lactose, maltose, sucrose, trehalose, melibiose, D-melezitose, and raffinose.

Hemagglutination by the coelomic fluid preparations from *A. crassispina* and *H. pulcherrimus* was not inhibited by the tested sugars. No hemagglutination was observed in the presence of the sugar alone. On the other hand, hemagglutination by *P. depressus* was affected by nine sugars; D-galactose, L-glucose, D-digitose, inositol, salicin, lactose, melibiose, D-melezitose and raffinose. Complete inhibition was observed in the concentration range of 0.0125–0.05 M. These results suggested that there might be some correlation between the chemical nature and inhibiting activity in these 9 effective sugars. Inhibition tests with these sugars were made repeatedly and the results were reproducible.

Inhibition test with human saliva

It has already been reported that certain hemagglutinins from marine sources react with human erythrocytes, and hemagglutination by them can be inhibited by human secretor saliva (Brain and Grace, 1968; Pemberton, 1971). In connection with this fact, the effect of human saliva on hemagglutination by the coelomic fluid preparation of sea urchin was examined. The test method was as follows: An equal volume of saliva and the coelomic fluid preparation was incubated at 20° C for 2 hours, rabbit erythrocyte suspension was added, and re-incubated at 20° C for 30 minutes. All of 7 kinds of saliva used (2 of blood group A, 2 of blood group B, and 3 of blood group O) completely inhibited hemagglutination by the fluid of *A. crassispina*, but not those of *P. depressus* and *H. pulcherrimus*. When human erythrocytes instead of rabbit cells were used, hemagglutination by the fluids from three different species of sea urchin was completely inhibited by the saliva materials so far examined. Moreover, inhibitory effect of saliva was observed in agglutination of dog erythrocytes by *A. crassispina* but not by *H. pulcherrimus*, while the saliva slightly inhibited agglutination of guinea pig erythrocytes by *P. depressus* but not by *H. pulcherrimus*. In all these cases, inhibition of hemagglutination by saliva seems not to be related to the difference in blood group of man, and saliva itself had no effect on erythrocytes.

Absorption test

The specificity of hemagglutinin of sea urchin was examined by the absorption method. Tests were scheduled to determine whether the hemagglutinin for rabbit

TABLE IV

Specificity of hemagglutinin

Sea urchin	Erythrocyte used for absorption	Titers of hemagglutinin to			
		Man	Rabbit	Dog	Guinea pig
A. crassispina	No absorption	2^4	2^8	2^5	<2
	Man	<2	<2	<2	—
	Rabbit	<2	<2	<2	—
	Guinea pig	2^2	2^8	2^4	—
	Sheep	2^3	2^8	2^4	—
P. depressus	No absorption	2^5	2^8	<2	2^5
	Man	<2	2^6	—	<2
	Rabbit	<2	<2	—	<2
	Guinea pig	<2	2^6	—	<2
	Sheep	2^3	2^6	—	2^3
H. pulcherrimus	No absorption	2^8	2^{11}	2^9	2^7
	Man	<2	2^9	2^9	2^6
	Rabbit	2^6	<2	<2	<2
	Guinea pig	2^6	2^9	2^8	<2
	Sheep	2^6	2^9	2^8	2^5

erythrocytes was identical to those for others, human, dog or guinea pig erythrocytes, and whether the hemagglutinin for each erythrocytes of the animals was identical to that for rabbit cells. The coelomic fluid preparation from sea urchin was mixed with an equal volume of packed erythrocytes. The mixture, after placing at 20° C for 2 hours, was centrifuged and then the supernatant was used for the hemagglutionation test.

These results are shown in Table IV. It is obvious that the hemagglutinin for rabbit erythrocytes was eliminated through the absorption by human erythrocytes in *A. crassispina*, and *vice versa*. Either rabbit cells or human cells absorbed the hemagglutinin for dog erythrocytes. Therefore, the hemagglutinin of *A. crassispina* is thought to be homogeneous. On the other hand, two hemagglutinins may occur in the coelomic fluid preparation from *P. depressus*. One is only to rabbit erythrocytes and another is common to rabbit, human and guinea pig erythrocytes. It may be assumed that each of these two hemagglutinins reacts to a different site on the surface of rabbit erythrocytes. In the case of *H. pulcherrimus*, three hemagglutinins may occur in this fluid preparation; the hemagglutinin only for human erythrocytes, the hemagglutinin being common to rabbit, dog and guinea pig erythrocytes, and the hemagglutinin being common to rabbit and dog erythrocytes.

In addition, no difference in absorption tests was observed among the types of blood group (A, B, O) in man, and the erythrocytes which could not be agglutinated by the coelomic fluid preparation from sea urchin did not absorb the hemagglutinin of sea urchin.

DISCUSSION

Present results showed that three species of sea urchin possessed hemagglutinins for several mammalian erythrocytes in their coelomic fluid. Rabbit erythrocytes

showed the highest susceptibility to three coelomic fluid preparations so far examined. In this case, calcium ion was essential for development of high hemagglutinating potency and the addition of EDTA abolished this activity. Magnesium ion was entirely ineffective. As one of the procedures for the examination of hemagglutinin activity in invertebrates, the use of calcium has been proposed in order to detect the activity (Uhlenbruck, Reifenberg and Heggen, 1970). Marchalonis and Edelman (1968) reported that a hemagglutinin from the hemolymph of the horseshoe crab, *Limulus polyphemus*, is a protein having a molecular weight of approximately 40×10^4, which consists of a number of subunits linked through non-covalent interactions, and that its agglutinating activity is potentiated by calcium but not by magnesium. They suggested that bound calcium may stabilize the native structure of the protein. As described in this paper, some difference was observed in the response of hemagglutinins from the three species of sea urchin to chemical and physical agents. The hemagglutinins from *A. crassispina* and *P. depressus* might be a protein-like substance, because they were fairly unstable to heat treatment, and to bromelain and 2-mercaptoethanol. However, it is unknown that the difference in the effects of bromelain and trypsin is due to either the susceptibility of active sites of hemagglutinin to both enzymes or the necessity of certain molecular size of agglutinin to induce hemagglutination. Their molecular weight was also determined as over 20×10^4 by means of gel filtration on Sephadex G-200 (unpublished data). Considering these facts it seems likely that calcium ion participates in stabilization of native structure of the hemagglutinins, as in the hemagglutinin of horseshoe crab mentioned above. Meanwhile, from the fact that hemagglutinating activity of *H. pulcherrimus* was heat-stable and resistant to proteinase and 2-mercaptoethanol, the active principle was considered to be complicated carbohydrates. Fuke and Sugai (1972) have recently reported that the hemagglutinin of ascidian may be a polysaccharide or a mucopolysaccharide from the fact that it is very stable to heat, is resistant to trypsin digestion, and is destroyed by periodate. The hemagglutinin from *H. pulcherrimus* was very similar in properties to that of ascidian except for resistance against periodate treatment.

Watkins and Morgan (1952) showed first that simple sugars are capable of neutralizing hemagglutinin of an eel. Later, Mäkelä (1957) reported the inhibition of hemagglutination by plant agglutinins (Leguminosae seeds) with simple sugars, and classified the sugars into four groups according to the correlation between plant agglutinin and simple sugars. The hemagglutinin of sea urchin (*A. crassispina* and *H. pulcherrimus*) against rabbit erythrocytes was not inhibited with the simple sugars tested while the hemagglutination by the fluid from *P. depressus* was inhibited by several simple sugars. The inhibition in the case of *P. depressus* was never dependent on Mäkelä's classification. In addition, it is known that simple sugars inhibit only a few animal agglutinins and that plant agglutinins in general neutralize at lower concentrations of simple sugars than animal agglutinins (Mäkelä, 1957; Pemberton, 1969). Since the inhibitory effect of L-fucose was considerably less than the activity of the intact H-substance, Watkins and Morgan (1952) stated that simple sugar alone does not account for the complete specificity of the blood group hapten. Therefore, it might be thought that the receptor site for the hemagglutinin of sea urchin was more complex than that for simple sugars.

The hemagglutinin in coelomic fluid preparation from sea urchin could be

absorbed by erythrocytes. This property is similar to that of isohemagglutinin present in mammalian serum. The data of absorption test showed that the hemagglutinin of *A. crassispina* was homogeneous to the erythrocytes tested, and that in the coelomic fluid preparation from *P. depressus* two hemagglutinins occurred. Furthermore, three hemagglutinins could be, at least, found in the coelomic fluid preparation from *H. pulcherrimus*. On the other hand, there was a parallelism between the results of absorption test and those of saliva inhibition test so far examined.

Brown, *et al.* (1968) reported the hemagglutinating activity of coelomic fluids from seven species of sea urchin against human erythrocytes. All of the fluids tested, except that from *Diadema antillarum*, reacted with human cells (A, B, O group) while the fluid from *Strongylocentrotus drobachiensis* did not react with A_1 and O_h (Bombay) cells. In the case of fluids from *Echinarachnius parma* and *S. drobachiensis*, a difference in hemagglutinating activity was found among individuals of specimens; three of 6 in *E. parma* and 5 of 15 in *S. drobachiensis* showed the activity. This is in agreement with the present data that there was a difference in the activity of specimens from *A. crassispina*. The difference in individuals was also reported on hemagglutination by the sera of horseshoe crab (Cohen, Rose and Wissler, 1955). In a previous paper (Ryoyama, 1973) it was shown that the coelomic fluid of sea urchin possessed hemolysin, which was not identical with hemagglutinin, and that the difference of hemolytic activity in individuals was seen, as in hemagglutinating activity reported in this paper. There was no correlation between the occurrence of hemolysin and hemagglutinin in each of the specimens. Brown, *et al.* (1968) also detected hemolytic activity in the fluid from *Lytechinus variegatus* but not in other species which belong to the same order as species we tested.

Tyler (1946) could not detect the hemagglutinating activity in the body fluid of *Strongylocentrotus purpuratus* against guinea pig erythrocytes. It may be thought that this is due to the difference of individuals in the species, or to deficiency of calcium ion in the reaction mixture. Calcium ion was essential for agglutination of guinea pig cells as well as other erythrocytes tested with *H. pulcherrimus*, as presented in this paper, and *S. purpuratus* belongs to the same family as *H. pulcherrimus* in the sea urchin. Therefore, calcium ion seems to be necessary for the hemagglutinating activity of the fluid from *S. purpuratus*.

On the other hand, Uhlenbruck, Sprenger and Heggen (1970) reported that the coelomic fluid from *Psammechinus miliaris* agglutinates human and animal erythrocytes, and that the preparation extracted from this fluid with phenol-KCl reacts with neuraminidase-treated human erythrocytes and pronase-treated cells of bovine and pigeon. Although sheep erythrocytes were not susceptible to the fluids of sea urchins tested, they became susceptible to the fluid of *A. crassispina* when the cells were treated with bromelain but not when treated with trypsin or cholera filtrate (crude neuraminidase). Agglutination of the enzyme-treated sheep erythrocytes is probably due to the unmasking of the receptor site on the surface of erythrocytes (Prokop, *et al.*, 1968).

Johnson (1969, 1970) has extensively investigated the coelomocytes of sea urchins; a similarity of the separating pattern in cellulose acetate-membrane electrophoresis was found between the coelomic fluid and the extracts of coelomocytes, and the results are influenced by the season of collecting the specimens. Further he

stated that "vibratile cell" (one of coelomoyctes) diffuses specific macromolecules into the fluid in response to a stress factor. The correlation between coelomic fluid and the extracts of coelomocytes on hemagglutinating activity is obscure.

Hemagglutinins have been demonstrated in several orders of sea urchin (mainly order Camarodonta), and therefore, it would be of interest to compare the hemagglutinins of each species of sea urchin chemically and biologically. At present, further investigations on the chemical properties of the hemagglutinin from *H. pulcherrimus* are in progress.

The author expresses many thanks to Prof. S. Koshimura for constant guidance in the course of this work, and to Dr. H. Ohtake of Hokuriku Serum Institute for kindly supplying human erythrocytes. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Japan.

Summary

The occurrence of natural hemagglutinins in the coelomic fluid of sea urchins, *Anthocidaris crassispina*, *Pseudocentrotus depressus*, and *Hemicentrotus pulcherrimus*, was reported.

The coelomic fluid preparations from three species of sea urchin were found to have hemagglutinating activity against rabbit and human erythrocytes but not against mouse and sheep erythrocytes so far examined. Dog erythrocytes were sensitive to the coelomic fluids of *A. crassispina* and *H. pulcherrimus*. The coelomic fluids of *P. depressus* and *H. pulcherrimus* also agglutinated guinea pig erythrocytes.

The hemagglutinins against rabbit erythrocytes from *A. crassispina* and *P. depressus* might be a protein or a protein-like substance and were heat-unstable and resistant to trypsin digestion, but not to bromelain, and were affected by 2-mercaptoethanol though the activity of the former was recovered when 2-mercaptothanol was removed. On the other hand, the hemagglutinin of *H. pulcherrimus* might be a large molecular, complicated carbohydrate, which was heat stable and resistant to trypsin, bromelain, 2-mercaptoethanol, and periodate.

The hemagglutinating activity of the coelomic fluids was greatly enhanced by the addition of calcium but not magnesium, and the hemagglutination by them was not dependent on temperature. Inhibition tests with simple sugars on hemagglutinating activity of the coelomic fluids resulted in failure to determine the specificity of receptor sites for these hemagglutinins. In addition, the results of absorption test on the specificity of the hemagglutinin of sea urchin were discussed.

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